The Time-Course of the Synthesis of Adenosine Triphosphate in Ehrlich Ascites-Tumour Cells, and the Effect of 2,4-Dinitrophenol

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1. In Ehrlich ascites-tumour cells kept in nitrogen at 20° for 20–30 min., the ATP concentration falls from about 15 μ moles/g. dry wt. to 2–3 μ moles/g. dry wt. 2. If oxygen is admitted to such cells, the ATP concentration rises again in 1–2 min. to about 15 μ moles/g. dry wt. 3. If glucose is added, in nitrogen, there is a slower increase in ATP concentration to about 6 μ moles/g. dry wt., followed by a fall and then by a still slower rise. 4. With glucose and oxygen, the ATP concentration rises rapidly in 1 min. to about 8 μ moles/g. dry wt., then falls, and finally increases slowly to reach 15 μ moles/g. dry wt. in 2 hr. 5. 2,4-Dinitrophenol (0.3 mm) has little effect on these processes. 6. At 1.0 mm, 2,4-dinitrophenol completely inhibits the ATP synthesis dependent on the endogenous respiration, while leaving that in the presence of glucose only a little impaired in rate, and considerably greater in magnitude. 7. ATP synthesis in the presence of glucose and 1.0 mm 2,4-dinitrophenol is about three times as fast in oxygen as in nitrogen.

Information about the generation of ATP in cells has been obtained chiefly by the study of mitochondria and their fragments. Such information from intact cells can be obtained directly only if the ATP concentration is first lowered by anaerobiosis (Laws & Stickland, 1962, 1963), when the subsequent regeneration of ATP under various experimental conditions can be observed. Convenient materials for this purpose are Ehrlich ascites-tumour cells (Laws & Stickland, 1962) and yeast (J. O. Laws & L. H. Stickland, unpublished work).

In Ehrlich ascites-tumour cells the important part of ATP regeneration occurs during the first few minutes. In this period the utilization of glucose is anomalous (Ibsen, Coe & McKee, 1958; Coe, 1966; Coe, Ibsen, Dixon & McKee, 1966): glucose disappears rapidly in the first minute, without identified products, and then no more glucose disappears until the steady-state glycolysis sets in (after 4 min. at 37°, or after about 10 min. at 25°).

During some experiments designed for another purpose, in which 2,4-dinitrophenol was used as a control because it is known to uncouple oxidative phosphorylation, it was observed that this reagent (at 0.3 mm) had little effect on either the rate or the extent of this synthesis of ATP, and this was sufficiently odd to be worthy of closer study.

METHODS

Ascites-tumour cells. The Ehrlich ascites tumour [a strain originated by Klein (1951) from a solid Ehrlich carcinoma] was maintained in mice of the BalbC strain, and grown for use either in mice of this strain or in C3H mice. The pooled ascites from up to 60 mice was cooled in ice, filtered through a plug of glass wool and kept at 0° until the beginning of the experiment. To prevent clotting, heparin was added (about 1 unit/ml. of ascites). Washing of the cells was omitted for the reasons given by Laws & Stickland (1963).

Control of pH. No buffer was added. The pH of the fresh ascites was 7.3–7.4, and the fluid was so heavily buffered with phosphate and protein that no change in pH could be detected (phenol red) at the end of the experiments.

Determinations. Respiration was measured in air by Warburg manometry, glucose by the method of Hanes (1929) and P_i by the method of Fiske & Subbarow (1925).

ATP was determined by (a) an ion-exchange resin column method based on that of Cohn & Carter (1951), as described by Laws & Stickland (1963) (this method gave also a value for ADP), and (b) the enzymic method of Hohorst, Kreutz & Bücher (1959), by using hexokinase and glucose 6-phosphate dehydrogenase and measuring the reduction of NADP⁺. With known amounts of ATP, method (a) gave almost quantitative yields, method (b) a 70% yield; results by the latter method were corrected by this factor.

In a medium with a high protein concentration, the intracellular 2,4-dinitrophenol concentration might be lower than the overall concentration. Samples of ascites were treated with a series of concentrations of 2,4-dinitrophenol and

centrifuged. Samples of the supernatants were taken and freed from protein by trichloroacetic acid; the volume of the cell fraction was measured, and, after the tubes had been freed as far as possible from supernatant, the whole cell fraction also treated with trichloroacetic acid. In the protein-free solutions (supernatants and cell extracts) 2,4-dinitrophenol was estimated in alkaline solution by colorimetry at 360 m μ . To determine the extent of contamination of the cell fraction with supernatant, similar experiments were carried out with an indicator known not to penetrate cells (thymol blue was used), which was estimated in alkaline solution at 585 m μ . For comparison, a few measurements were made on washed cells. The ascites was centrifuged, and the cells were washed twice with about 10 vol. of Ca²⁺-free Krebs-Ringer solution (Burris, 1957) and finally made up to the volume of the original ascites.

Lactate was determined in some experiments by the method of Barker & Summerson (1941) as modified by LePage (1957), but more frequently by that of Hohorst (1963).

For determination of dry weight, samples of each suspension were washed twice with Ca²⁺-free Krebs-Ringer solution (Burris, 1957) and dried at 100°, a correction being made for the salt content of the medium. In normal ascites the content of cells was between 29 and 39 mg. dry wt./ml., and the concentration in the reaction mixtures was therefore about 30 mg. dry wt./ml.

To obtain the intracellular concentration of P_i, a sample of ascitic fluid free from cells was treated with trichloroacetic acid, and P_i was estimated in the supernatant. The appropriate correction was applied to the P_i estimations on the whole-cell suspensions.

Experimental procedure. The experiments described below required that nine separate reaction mixtures should be prepared, and, since all nine could not be sampled simultaneously, they were staggered to the extent that the period of rapid sampling from each took place during the period of less frequent sampling from the preceding ones.

The appropriate volume of ascites for each experimental procedure was measured into a 250 ml. Erlenmeyer flask and the temperature rapidly brought to 20° while a fast stream of O₂-free N₂ was passing through the flask. The flask was then corked and left at room temperature (20 ± 2°). At intervals of about 10 min. the other flasks were similarly treated.

After a preliminary 20 or 30 min. of anaerobiosis, the flasks were placed in turn in a water bath at 25° and shaken. The appropriate volume of 2,4-dinitrophenol solution at pH 7.4 was added to give overall concentrations of 0.3 or 1.0 mm [or the same volume of Ca²⁺-free Krebs-Ringer solution (Burris, 1957) in the controls] and a zero sample taken. The reaction was started by the addition of O₂, or of glucose (sufficient 4% solution to give a final concentration of 0.2%), or of both simultaneously. Samples were then taken into trichloroacetic acid or HClO₄ at intervals of 30 sec. up to 2 min., and thereafter less frequently up to 40 min., and centrifuged within 1 hr. of sampling.

The experiments were carried out in two series. In the first, respiration, glucose utilization, changes in P_i concentration and changes in ATP and ADP concentrations (measured by the ion-exchange resin method) were studied, and the effect of 0.3 mm- and 1.0 mm-2,4-dinitrophenol on them. In the second, respiration, glucose utilization, changes in ATP concentration (measured by the enzymic method) and changes in lactate concentration were studied, and the effect

on them of 1.0 mm-2,4-dinitrophenol and 0.2 mm-iodoacetate. So far as the two series overlapped, the results were practically identical.

RESULTS

Intracellular 2,4-dinitrophenol concentration.

Table 1 shows the effect of the protein in the ascitic fluid on the distribution of 2,4-dinitrophenol between cells and medium. The intracellular concentrations were lower than the overall concentrations, ranging from one-third at 1.0 mm to one-fifth at 0.1 mm. The extracellular concentrations were slightly raised. With washed cells this effect was absent, the 2,4-dinitrophenol being slightly more concentrated in the cells than outside. The presence of glucose in the medium made no difference to the distribution of 2,4-dinitrophenol. In the experiments described below, the concentration of 2,4-dinitrophenol given is the overall concentration.

Glucose utilization. The course of glucose utilization by cells metabolizing in ascitic fluid was the

Table 1. *Distribution of 2,4-dinitrophenol between Ehrlich ascites-tumour cells and medium at 25°*

The numbers of determinations are given in parentheses.

Overall	Concn. of dinitrophenol (mm)		
	Unwashed cells		Washed cells* Intracellular
	Intracellular	Extracellular	
1.0	0.36 (7)	1.06 (7)	1.06 (2)
0.6	0.14 (2)	0.67 (1)	—
0.3	0.064 (6)	0.33 (6)	0.37 (2)
0.1	0.018 (4)	0.118 (5)	0.11 (2)

* Samples of cells washed free from ascitic fluid (see the Methods section).

Table 2. *Extent of the rapid initial disappearance of glucose in Ehrlich ascites-tumour cells, and the effect of 2,4-dinitrophenol and sodium iodoacetate, at 25°*

The numbers of observations are given in parentheses.

Inhibitor	Glucose utilized after 4 min. (μ moles/g. dry wt.)	
	In N ₂	In O ₂
None (control)	76 (35)	60 (35)
Dinitrophenol (1.0 mm)	81 (25)	82 (25)
Iodoacetate (0.2 mm)	39 (12)	42 (12)
Dinitrophenol (1.0 mm) + iodoacetate (0.2 mm)	52 (7)	58 (7)

Table 3. *Effect of 2,4-dinitrophenol and sodium iodoacetate on the rate of glucose utilization by Ehrlich ascites-tumour cells during the linear phase (from 10 min. after the addition of glucose) at 25°*

The results are the averages of six experiments. The Pasteur quotient is the rate of disappearance of glucose in O_2 /rate in N_2 .

Inhibitor	Rate of glucose disappearance (μ moles/g. dry wt./hr.)		Pasteur quotient
	In N_2	In O_2	
None (control)	225	135	0.61
Dinitrophenol (0.3 mM)	275	300	1.12
Dinitrophenol (1.0 mM)	295	445	1.54
Iodoacetate (0.2 mM)	0	0	—

Fig. 1. Rate of production of lactate from glucose by Ehrlich ascites-tumour cells at 25°. (a) Control; (b) +2,4-dinitrophenol (1.0 mM); (c) +iodoacetate (0.2 mM); (d) +2,4-dinitrophenol (1.0 mM) + iodoacetate (0.2 mM). Δ , In O_2 ; $\blacktriangle$, in N_2 . The broken lines show for comparison the glucose utilization. Lactate is plotted as lactate/2, to show the relationship to the glucose used.

same as that found by Ibsen *et al.* (1958) with washed cells, namely a rapid disappearance in 1 min., followed by a period of quiescence up to about 10 min., when the steady-state linear utilization of

glucose started. The experiments of Ibsen *et al.* (1958) were exclusively aerobic; we found in addition that anaerobically a similar course was followed, the initial rate being lower (about two-thirds of the aerobic rate), and the amount of glucose used being slightly higher (Table 2). After about 10 min. the steady-state utilization of glucose started and continued linearly until the glucose was depleted; the usual Pasteur effect was observed (Table 3).

At 0.3 mM 2,4-dinitrophenol had no effect on the initial phase, but inhibited the Pasteur effect in the steady state (Table 3). At 1.0 mM 2,4-dinitrophenol slightly increased the amount of glucose used in the initial phase (Table 2) and further augmented the rate of disappearance aerobically in the steady state (Table 3).

Lactate production. Fig. 1 shows the lactate production in the initial phase and in the beginning of the steady state. In the steady state, lactate production ran very nearly parallel with glucose disappearance (Figs. 1a and 1b). When iodoacetate was present, no lactate appeared (Fig. 1c and 1d). In the initial stages, as was shown by Ibsen *et al.* (1958) in experiments in oxygen, lactate production lagged behind glucose utilization. This was also true in nitrogen (Fig. 1a) and in the presence of 2,4-dinitrophenol (Fig. 1b).

Respiration. Table 4 gives the effects of glucose and of 2,4-dinitrophenol on the respiration of unwashed Ehrlich ascites-tumour cells at 25°. It shows the absence of serious inhibition by 2,4-dinitrophenol in the initial stages, but after the first hour there was considerable inhibition, especially in the presence of glucose (cf. Cereijo-Santalo, 1962).

Changes in intracellular P_i concentration. In the control experiments with glucose present, almost all the intracellular P_i was esterified very rapidly, about three times as fast in oxygen as in nitrogen (Table 5), decreasing the concentration from about 100 μ moles/g. dry wt. to about 10 μ moles/g. dry wt. The concentration of P_i then gradually rose somewhat. In the absence of glucose, in oxygen, the diminution was much less (to about 70 μ moles/g. dry wt.) and slower than with glucose present, and this concentration remained constant from 4 min. to 40 min.

At 0.3 mM 2,4-dinitrophenol had little effect on the shape of the curves, but slowed down by about 30% the rate in the presence of glucose and oxygen, with a smaller effect on the anaerobic rate (Table 5).

At 1.0 mM 2,4-dinitrophenol slowed still further the rates in the presence of glucose (Table 5), though not greatly affecting the ultimate concentrations reached, but entirely abolished the disappearance of P_i associated with the endogenous respiration.

Table 4. *Effect of 2,4-dinitrophenol and sodium iodoacetate on the respiration of Ehrlich ascites-tumour cells, respiring in ascitic fluid, in the presence and absence of glucose, at 25°*

Inhibitor	No. of expts.	Rate of respiration (μ l. of O ₂ absorbed/mg. dry wt./hr.)					
		Glucose absent			Glucose present		
		0-1 hr.	1-2 hr.	2-3 hr.	0-1 hr.	1-2 hr.	2-3 hr.
None (control)	5	3.65	3.40	3.33	2.51	2.30	2.38
Dinitrophenol (0.3 mm)	5	5.60	4.62	4.26	3.72	1.66	0.81
Dinitrophenol (1.0 mm)	5	2.68	1.64	1.02	1.79	0.53	0.36
None (control)	2	4.02	4.02	3.83	2.55	2.77	2.80
Dinitrophenol (1.0 mm)	2	3.74	2.14	1.17	2.88	0.98	0.83
Iodoacetate (0.2 mm)	2	3.93	3.61	2.85	1.66	1.62	1.32
Dinitrophenol (1.0 mm) + iodoacetate (0.2 mm)	2	4.03	1.89	0.84	3.50	1.44	0.80

Table 5. *Relative initial rates of disappearance of intracellular P_i in Ehrlich ascites-tumour cells, in the presence and absence of oxygen, glucose and 2,4-dinitrophenol, at 25°*

The results are the averages of four to six experiments. The faster reactions were not linear even for 1 min., so the rates given are only approximations.

Concn. of dinitrophenol (mm)	Rate of diminution of P _i (μ moles/g. dry wt./hr.)			Ratio of rates $\frac{N_2 + \text{glucose}}{O_2 + \text{glucose}}$
	In O ₂ (no added substrate)	In O ₂ (+glucose)	In N ₂ (+glucose)	
0	1200	3950	1400	0.36
0.3	1250	2600	1250	0.48
1.0	0	2100	950	0.45

Table 6. *Rate of resynthesis of ATP by Ehrlich ascites-tumour cells at 25°, in the presence and absence of oxygen, glucose, 2,4-dinitrophenol and sodium iodoacetate*

The results are the average of four to six experiments.

Inhibitor	Initial rate of ATP synthesis (μ moles/g. dry wt./hr.)			Ratio of rates $\frac{N_2 + \text{glucose}}{O_2 + \text{glucose}}$
	In O ₂ (no added substrate)	In O ₂ (+glucose)	In N ₂ (+glucose)	
None (control)	340	220	65	0.30
Dinitrophenol (0.3 mm)	265	210	70	0.33
Dinitrophenol (1.0 mm)	0	170	45	0.26
None (control)	405	245	105	0.43
Dinitrophenol (1.0 mm)	0	245	110	0.45
Iodoacetate (0.2 mm)	420	120*	0	0
Dinitrophenol (1.0 mm) + iodoacetate (0.2 mm)	0	40*	0	0

* The concentration of ATP reached a maximum at 2 min., and then fell off.

Changes in ATP concentration. In the control experiments the only condition under which rapid and complete resynthesis of ATP was observed was when oxygen alone was added to the depleted cells,

i.e. the resynthesis associated with the endogenous respiration. The original concentration of ATP was restored within 2 min. at 25° and maintained unchanged up to 40 min. (Tables 6 and 7). When

Table 7. Concentrations of ATP in Ehrlich ascites-tumour cells, during the linear phase of glucose consumption (10 min. to 40 min.) in the presence and the absence of oxygen, glucose and 2,4-dinitrophenol, at 25°

The results are the averages of four to seven experiments.

Concn. of dinitrophenol (mm)	Time after start of expt. (min.) ...	Concn. of ATP (μ moles/g. dry wt.)			
		0	10	25	40
0	In O ₂	2.4	12.9	13.3	13.0
	In O ₂ +glucose	2.3	7.1	8.7	9.9
	In N ₂ +glucose	2.2	5.4	6.7	8.0
0.3	In O ₂	2.1	11.8	12.0	12.1
	In O ₂ +glucose	2.3	10.1	11.1	12.5
	In N ₂ +glucose	2.1	5.8	7.4	8.2
1.0	In O ₂	2.4	2.3	1.9	1.7
	In O ₂ +glucose	2.3	10.9	10.3	6.8
	In N ₂ +glucose	2.0	7.1	8.3	8.6

glucose was added in the presence of oxygen, a similar rapid synthesis of ATP was seen in 1 min., but only to about half the original concentration. This was succeeded by a fall in the period 1–4 min., during which up to one-half of the rapidly synthesized ATP was lost, and this again by a steady slow rise (Table 7). When glucose was added in the continued presence of nitrogen, ATP synthesis was smaller and slower than with glucose in oxygen, and the concentration fell as in oxygen and then rose steadily, but never attained more than 60% of that in the 'endogenous' experiment (Table 7).

With 0.3mm-2,4-dinitrophenol the 'endogenous' synthesis of ATP, though slightly slower (Table 6), was as complete as in the control and was maintained for 40 min. (Table 7). With glucose in oxygen, the initial increase was greater, the fall between 2 and 4 min. was less and the final rise quicker and more complete than in the control (Tables 6 and 7). With glucose in nitrogen, the initial rapid rise was greater, the fall smaller and the final slow rise quicker than in the control; the final concentration reached was not higher than in the control (Tables 6 and 7).

With 1.0mm-2,4-dinitrophenol the 'endogenous' ATP synthesis was completely suppressed (Table 6). With glucose in oxygen the initial ATP synthesis, though slower again (Table 6), proceeded roughly linearly for 3 min. until 80% of full resynthesis was reached; this concentration was maintained until 25 min., when it began to fall (Table 7).

The above results were obtained by the ion-exchange method for ATP estimation. With the enzymic method almost identical results were

Fig. 2. Early stages in the change of concentration of ATP in Ehrlich ascites-tumour cells, in the presence and absence of oxygen, glucose, 2,4-dinitrophenol and iodoacetate. (a) Control; (b) +2,4-dinitrophenol (1.0mm); (c) +iodoacetate (0.2mm); (d) +2,4-dinitrophenol (1.0mm)+iodoacetate (0.2mm). □, In O₂ (no added substrate); Δ, in O₂+glucose; ▲, in N₂+glucose. ATP estimation was by the enzymic method.

found (Table 6). One such experiment is shown in Fig. 2, giving only the early stages.

Effect of iodoacetate. Sodium iodoacetate at 0.2 mm, the lowest concentration to cause complete inhibition of steady-state glucose utilization, had no effect on the endogenous respiration in the first hour, nor on the ATP synthesis coupled with the latter. It completely abolished anaerobic ATP formation in the presence of glucose. In the presence of glucose and oxygen, instead of restoring the 'endogenous' ATP synthesis, it allowed the ATP concentration to rise only to a very small peak at 2 min. (Fig. 2) followed by a return to the original level. The presence of 2,4-dinitrophenol in addition to iodoacetate gave the same result, apart from the abolition of the 'endogenous' ATP synthesis.

Changes in ADP concentration. The concentration of ADP, initially about 6 μ moles/g. dry wt., remained constant between 10 min. and 40 min. at values between 4 and 5 μ moles/g. dry wt., unaffected by 2,4-dinitrophenol.

Quantitative relationship between P_i used and ATP

formed. In the presence of glucose, large amounts of P_i are taken up into phosphorylated intermediates of glucose metabolism, and no particular relationship between P_i disappearing and ATP being formed is to be expected. Actually, some 30% of the P_i that disappears is accounted for as ATP.

In the absence of glucose, it is not known what phosphorylated intermediates may occur and accumulate in the oxidation of the unknown substrate(s) of endogenous respiration. The average of nine experiments showed 30.9 μ moles of P_i disappearing/g. dry wt. and 12.9 μ moles of ATP appearing/g. dry wt., so that 84% of the P_i is accounted for as ATP.

During the first 2 min. after the addition of glucose, glucose and P_i disappear *pari passu* and, allowing for that part of the P_i used in ATP formation, in the stoichiometrical ratio 1:1.

DISCUSSION

The rate of regeneration of ATP, in cells in which its concentration has been lowered by a brief exposure to anaerobic conditions, has been studied (a) in cells respiring endogenously, (b) in cells respiring in the presence of glucose, and (c) in cells glycolysing glucose anaerobically. In both (a) and (c), ATP synthesis is the result of a single process (oxidation of endogenous substrates and glycolysis respectively), but in (b) three processes are present simultaneously, namely oxidation of endogenous substrates, oxidation of glucose and glycolysis. Bloch-Frankenthal & Ram (1959) deduced from experiments with [U - ^{14}C]glucose that roughly one-half of the oxygen consumption under these conditions represents oxidation of glucose; this rate of oxygen consumption is about 90 μ moles/g. dry wt./hr. (Table 4), so the rate of oxidation of glucose is about 7 μ moles/dry wt./hr. or 5% of the total glucose utilization (140 μ moles/g. dry wt./hr.; Table 3), the rest being chiefly converted into lactate.

The initial stages of glucose utilization by Ehrlich ascites-tumour cells remain unexplained. Neither Ibsen *et al.* (1958) nor Hess & Chance (1961) accounted for the fate of the glucose rapidly removed in the first minute in oxygen (at 37°, corresponding to about 3 min. at 25°), nor the changes in the following few minutes before steady linear consumption of glucose set in. We have found that a similar course is followed in nitrogen, though the rate in the first minute is lower than in oxygen, i.e. there is a reversed Pasteur effect.

ATP resynthesis in our control experiments is rapid and complete only in association with the endogenous respiration. In the presence of glucose, both in nitrogen and in oxygen, we find a small rapid synthesis, followed by a fall and later a slow

increase, in oxygen up to the maximum concentration, in nitrogen only to a lower concentration. The initial burst of ATP synthesis must be related to the initial rapid glucose uptake (since it is seen anaerobically as well as aerobically); the fall marks presumably the onset of hexokinase activity, and the later rise the ATP synthesis due to glycolysis, and, in the presence of oxygen, also to glucose oxidation. The part played by the diminished endogenous respiration in this period is unexplained. The rate of this later rise in ATP concentration is of little significance, as it represents only the balance between synthesis and breakdown; only the initial rate is relatively unaffected by this consideration.

The results obtained with 2,4-dinitrophenol pose this problem: 1.0 mM-2,4-dinitrophenol (about 0.35 mM in the cells), although abolishing the ATP synthesis derived from the endogenous respiration, allows ATP synthesis in oxygen plus glucose to proceed at a slightly diminished rate (Table 6) but to a much higher concentration (Fig. 2) than in the absence of 2,4-dinitrophenol. This ATP synthesis in the presence of 2,4-dinitrophenol is not related to lactate formation, because it is three times as fast in oxygen as in nitrogen (Table 6), and over the same period of time lactate production is very small and no faster in oxygen than in nitrogen (Fig. 1).

The lowering of the intracellular concentration of 2,4-dinitrophenol in the presence of the proteins of the ascitic fluid was not unexpected; Aizawa (1961) had observed a diminished effectiveness of 2,4-dinitrophenol when proteins were added to the extracellular medium. The concentration required to inhibit 'endogenous' ATP synthesis (0.35 mM) remains very high compared with those used in experiments with mitochondria. The immediate entry of 2,4-dinitrophenol into the cells was shown by, e.g., Chance & Hess (1959), and in our own experiments by the immediate suppression of esterification of P_i and synthesis of ATP.

REFERENCES

- Aizawa, M. (1961). *Folia pharm. jap.* **57**, 193.
- Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
- Bloch-Frankenthal, L. & Ram, D. (1959). *Cancer Res.* **19**, 835.
- Burris, R. H. (1957). In *Manometric Techniques*, revised ed., p. 149. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
- Cereijo-Santalo, R. (1962). *Arch. Biochem. Biophys.* **98**, 86.
- Chance, B. & Hess, B. (1959). *J. biol. Chem.* **234**, 2416.
- Coe, E. L. (1966). *Cancer Res.* **26**, 269.
- Coe, E. L., Ibsen, K. H., Dixon, M. & McKee, R. W. (1966). *Cancer Res.* **26**, 276.
- Cohn, W. E. & Carter, C. E. (1951). *J. Amer. chem. Soc.* **72**, 4273.

- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 387.
- Hanes, C. S. (1929). *Biochem. J.* **23**, 99.
- Hess, B. & Chance, B. (1961). *J. biol. Chem.* **236**, 239.
- Hohorst, H.-J. (1963). In *Methods of Enzymatic Analysis*, p. 260. Ed. by Bergmeyer, H. U. New York and London: Academic Press Inc.
- Hohorst, H.-J., Kreutz, F. H. & Bücher, Th. (1959). *Biochem. Z.* **332**, 18.
- Ibsen, K. H., Coe, E. L. & McKee, R. W. (1958). *Biochim. biophys. Acta*, **30**, 384.
- Klein, G. (1951). *Exp. Cell Res.* **2**, 518.
- Laws, J. O. & Stickland, L. H. (1962). *Biochem. J.* **83**, 84.
- Laws, J. O. & Stickland, L. H. (1963). *Biochem. J.* **87**, 520.
- LePage, G. A. (1957). In *Manometric Techniques*, revised ed., p. 275. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.